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Assessing Healthspan in Long Lived C. elegans

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Abstract

"The plasticity of ageing suggests that longevity may be controlled epigenetically by specific alterations in chromatin state" (Greer and Brunet, 2010). Epigenetics, in a broad sense, is a bridge between genotype and phenotype—a phenomenon that changes the final effect of a locus or chromosome without changing the underlying DNA sequence. This project reviews research performed by Greer and Brunet, (2010), Hamilton et al., (2013), and Lee et al., (2008), that involved the use of RNAi to modify an epigenetic effect on the lifespan of the soil nematode *C. elegans.* RNA interference (RNAi) was used to regulate lifespan in *C. elegans* by causing deficiencies in methylation of histone H3 at lysine 4 (H3K4). While the previously mentioned authors observed extended lifespan in *C. elegans* treated with RNAi, neither study considered the quality of health of the animals with extended lifespan. Herein, methods used in previous studies were replicated to produce long-lived *C. elegans*, but the focus was quantifying the healthspan of the animals. Healthspan is a novel term in research that describes the length of a healthy life. When considering lifespan-extending treatments we must consider "healthspan"- the healthy adult period of unimpaired life that precedes functional decline (Herndon et al., 2002). A key question is whether the increasing proportion of the population surviving to advanced ages will display continued quality of existence, which can be defined as delayed on*set* of chronic illness and physical/mental decline. Experimental animals were classified into four categories that reflect health based on frequency of movement. Upon analysis of the data, it appears that mutations for genes *wdr*-5.1 and *set*-4, and RNAi-mediated knock down of *set*-2 produced an improved heatlhspan:lifespan ratio. However RNAi-mediated knock down of genes *wdr*-5.1, *set*-9, *set*-15, and *set*-18 did not improve the healthspan:lifespan ratio.

Introduction

Throughout history, humanity has been interested in the concept of "the fountain of youth". If a lifetime is limited, is it possible to prolong our period of life on Earth? Ageing research and medical treatment have sought to find means to extend lifespan, but at what point does physical decline significantly reduce the quality of life? While recent exciting discoveries have been made about the connection of genetics and the ageing process, it is unknown if advances in life extension technology are also accompanied by an increase in the extension of an organism's health and well-being.

Definition of Healthspan

Healthspan is a term that describes the length of a healthy life. When considering lifespan-extending treatments we must consider "healthspan"- the healthy adult period of unimpaired life that precedes functional decline (Herndon et al., 2002). Healthspan is a new focus in ageing research. While it is interesting to investigate methods of life extension, it is important to consider the quality of prolonged life. A key question is whether the increasing proportion of the population surviving to advanced ages will display continued quality of existence, which can be defined as delayed on*set* of chronic illness and physical/mental decline. An organism may possess longevity, but if it is frail and ill for a large portion of its late life, then perhaps life-extension is not a desirable pursuit. Ideally organisms that have an extended lifespan also have an extended healthspan. Research on life extension should aim to increase lifespan while decreasing time spent in a state of morbidity. There are several possible relationships between healthspan and lifesapn.

Possible Healthspan:Lifespan Relationship Scenarios

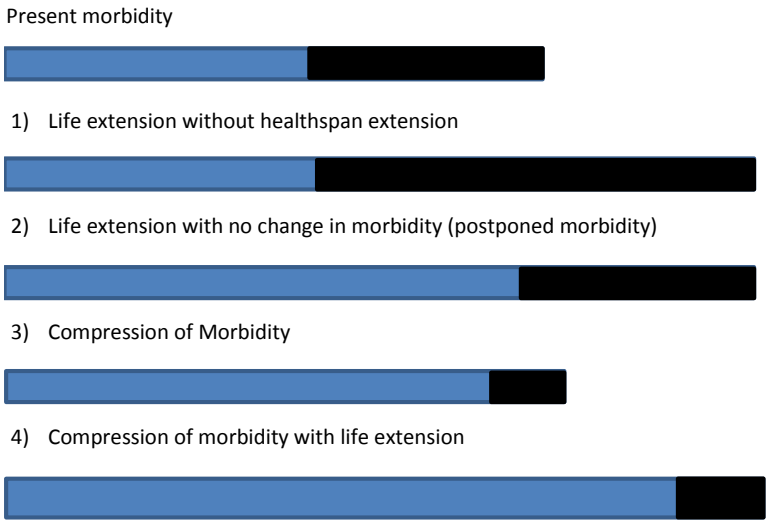


Figure 1: Concept adapted from (Kitzenberg, 2012). The bar represents lifespan with the black portion representing the duration of morbidity.

Figure 1 illustrates that when compared to present trends in morbidity, scenario 2) life extension with no change in morbidity, and scenario 4) life extension with compression of morbidity, are desirable outcomes for an extended lifespan with an improved healthspan: lifespan ratio. While lifespan was not extended in scenario 3) compression of morbidity, the healthspan:lifespan ratio was still improved. Organisms that demonstrate a delayed or shortened period of morbidity experience an improved healthspan. Obviously the most favorable outcome is to extend lifespan and decrease the time an organism spends in morbidity. Scenario 1), life extension with no healthspan extension is not a desirable outcome because the organism spends a larger portion of its life in a weakened state.

While the state of knowledge surrounding the connection between genetics and life-extension is expanding, little is known about the effects of a prolonged lifespan on healthspan. There has been hundreds of genome screenings performed to identify genes associated with ageing. An analysis of the results from three such research studies provided the background knowledge and methodology for this project (Greer and Brunet, (2010), Hamilton et al., (2013), and Lee et al., (2008)).Of the three studies, all identified genes that are shown to extend lifespan, but none considered healthspan.

List of Genes being investigated

Strain Name	**Gene**	**Study**	**Known phenotypes (other than life-extension)**
RB1304	*wdr*-5.1	Greer and Brunet (2010)	None.
RB1025	*set*- 2	Greer and Brunet (2010)	plays a role in germline development, postembryonic development, and RNA interference
MT14911	*set*- 4	Greer and Brunet (2010)	Lethal for allele tm1835; this phenotype was not found in three other RNAi screens, and is not observed for the probable null allele *set*-4(n4600)
MT16426	*set*-9	Greer and Brunet (2010)	Neither *set*-9(n4949) nor *set*-9(RNAi) has any obvious phenotypes.
RB2406	*set*- 15	Greer and Brunet (2010)	None
VC767	*set*-18	Greer and Brunet (2010)	Neither *set*-18(gk334) nor *set*-18(RNAi) has any obvious phenotypes.
RB777	*hcf*- 1	Hamilton et al. (2013) and Lee et al. (2008)	Negative regulator of adult lifespan. No other phenotypes are known.

Table 1: The table represents which studies investigated which genetic modifications of *C. elegans* chosen for this experiment.

Again, strains were selected because they were shown to produce long-lived animals in studies performed by Greer and Brunet, (2010), Hamilton et al., (2013), and Lee et al., (2008). Each strain represents a mutation in a particular genetic locus that increases lifespan. The right column of the chart includes information about other phenotypes besides longevity that are produced by the mutation. Strains RB1304 and MT14851 were obtained from The Caenorhabditis Genetics Center, in Minneapolis Minnesota. These strains represent *C. elegans* that are homozygous for the indicated mutant alleles. The remaining strains, RB1025, MT16426, RB2406, VC767, RB777, and an additional RB1304 represent genes that require the application of RNA interference (RNAi) to produce the long-lived phenotype, and were obtained from the Ahringer Library composed by Julie Ahringer's group at the Wellcome CRC Institute, University of Cambridge, Cambridge, UK.

Research Question

Certain genetic mutations that have been proven to extend lifespan in *Caenorhabditis elegans* may or may not also extend healthspan. The objective of this project is to test a *set* of seven genes (table 1) that have a known life-extending effect on the nematode, *C. elegans,* to assess if they also extend the healthspan of the animals. If genetically altered *C. elegans* display extended lifespan in combination with a proportionally extended healthspan, then the genetic treatment may be considered favorable.

C. elegans as an Animal Model

Several characteristics make *C. elegans* an excellent model in genetic research. *C. elegans* is a eukaryote, which carry many homologous genes to those of humans. It is estimated that about 60-80% of C. elegans genes have human homologs (Kaletta and Hengartner, 2006). Although *C. elegans* is a small, relatively simple organism it possess many tissue types in common with humans such as muscle, intestinal, and neural tissue. Because *C. elegans* has similar tissue types, it is possible to translate some findings from nematode research to higher organisms like humans.

The entire *C. elegans* genome has been sequenced. This feat was possible due to the relatively small size of the *C. elegans* genome which is about 9.7×10^7 base pairs compared to the human genome which is about 3 billion base pairs (Kaletta and Hengartner, 2006). Furthermore, manipulation of the genome can be easily accomplished by adding, removing, or altering specific genes through routine molecular genetic procedures.

Apart from its physiological similarity, *C. elegans* serve as a good animal model because it is easy to maintain in a laboratory. Hundreds of nematodes can be kept alive on a small plate of agar. It feeds on bacteria which is also easily stored and maintained. The average lifespan of *C. elegans* is 2-4 weeks, so it can be used for modeling the action of certain genes, environments, or treatments over multiple generations.

Another advantage of using *C. elegans* in genetic research is that the majority of any given population of worms are hermaphroditic. Because the worms self-fertilize, once homozygosity has been established for a certain gene, there is no change in the genetic make-up between individuals or

between generations unless a deliberate or natural mutation occurs. Because the worms are essentially identical clones, variations in behavior are minimized, making them very valuable in comparative genetic research.

A final reason why *C. elegans* is a particularly good candidate for RNAi research is that it is one of few organisms that display the phenomenon of "spreading". Thus far, *C. elegans* is one of few eukaryotic species known to produce a systemic response to the localized introduction of dsRNA (Descotte, 2003.) Although dsRNA may be introduced to only a few cells in the animal, the subsequent silencing effect occurs throughout the majority of the organism's cells. "Spreading" phenomenon is ideal because it demonstrates the effects of RNAi on a systemic level versus a localized one.

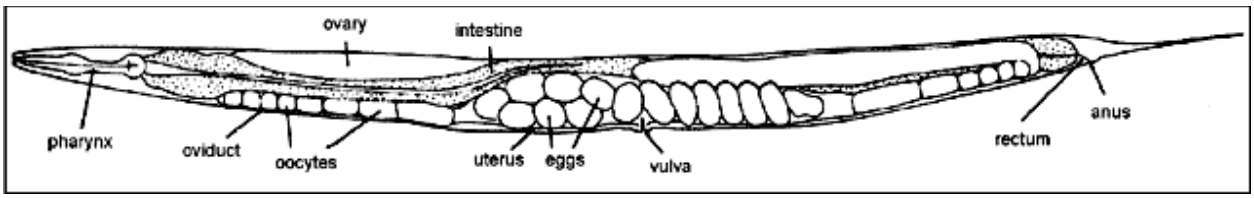


Fig. 2: Anatomy of *Caenorhabditis elegans*
http://avery.rutgers.edu/WSSP/StudentScholars/project/introduction/worms.html

Induction of Gene Knock Down through RNA interference

The majority of nematodes used in this project were fed *E.coli* bacteria containing a plasmid with an antisense RNA fragment to induce RNA interference (RNAi). When exogenous double stranded RNA molecules are introduced into a cell, a molecular response is generated to suppress expression of the gene with a nucleotide sequence complementary to the foreign RNA (Kammath et al., 2000). RNAi knocks down gene expression in two ways; by post-transcriptionally blocking protein production via mRNA, or through the modification of histone methylation at the transcriptional level in the genome. This project focuses on the actions of small interfering RNAi (siRNA) to initiate histone methylation to produce the desired life-extending result. Transcriptional RNAi silencing is initiated when dsRNA is cleaved in the cytoplasm by the ribonuclease like enzyme Dicer to generate small interfering RNA molecules that are about 22 nucleotides long (Bagasral et al., 2004). These small interfering RNAs (siRNAs) load onto an effector complex called RITS (RNA-induced transcriptional silencing complex) that contains an argonaute protein and targets complementary mRNAs associated with heterochromatin for inactivation (Verdel et al., 2004). RITS uses siRNA- dependent base pairing to guide association with either DNA or nascent RNA sequences at the target locus destined to be silenced, an association that is stabilized by direct binding to a methylated histone. RITS triggers heterochromatin formation in concert with well-known heterochromatin associated factors and directly links RNA silencing to heterochromatin modification (Allis et al., 2007).

The maintenance of heterochromatin regions by the RITS complex has been described as a self-reinforcing feedback loop, in which the RITS complex binds the methylated histones of a heterochromatin region and induces co-transcriptional degradation of any emerging messenger

RNA (mRNA) transcripts. The degraded mRNA fragments are then used as substrates in RNA-dependent RNA polymerase to replenish the complement of siRNA molecules (Sugiyama et al., 2005).

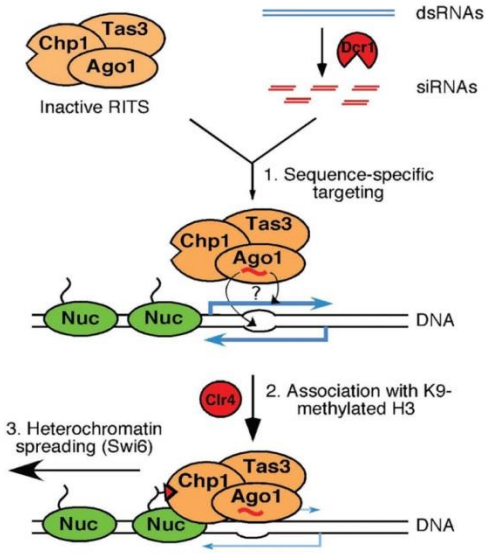


Figure 3: An example of the process of heterochromatin modification via RITS complex. (Verdel, 2004). In this project, histone residue H3K4 was studied.

Manipulation of Chromatin Modifiers Produces Effect

Histones are proteins found in the nuclei of eukaryotic cells that function to package long strands of DNA into structural units called nucleosomes (Allis et al., 2007). It is important to note that in *C. elegans*, direct DNA methylation does not occur and epigenetic modification must be accomplished via histone modification (Simpson et al., 1984). The assembly of nucleosomes creates chromatin which is the material from which chromosomes are composed. There are five specific families of histones, H1, H2(A), H2(B), H3, H4, and H5. Histones H2(A),H3(B), and H4 are known as core histones. H2A and H2B or H3 and H4 assemble to create a nucleosome core particle on which 147 base pairs of DNA wrap around (Luger et al., 1997). H1 and H5 histones act as linker proteins to bind the nucleosome at entry and exit sites and secure DNA in place on the core histone particle (Farkas, 2006).

Because histones are directly involved in the organization and packaging of DNA within the nucleus, modification of parts of a histone through acetylation, phosphorylation, and methylation can cause changes in regulation of gene function. Acetylation is correlated with an “open” chromatin configuration, and therefore with regions of DNA that are exposed and poised for transcription (Allis et al., 2007). Acetylation acts to neutralize the positive charge of a specific lysine site and reduce the binding strength of strongly basic histones or histone tails to negatively charged DNA. Although the mechanisms behind histone phosphorylation are not as well understood, histone phosphorylation similarly modifies chromatin to become transcriptionally active (Mahadevan et al., 1991). Methylation is unique because it can occur on both lysine and arginine, and can have positive or negative affect on transcriptional expression depending on the position of the residue (Allis et al., 2007). Also, there are three possible methylated states for each lysine residue, i.e. lysine can be mono-, di-, or trimethylated. Of particular interest, trimethylation of the histone H3 at lysine 4 (H3K4) is linked to activation of transcription (Allis et al., 2007). RNAi-mediated knock down of the *SET* domain of methyltransferases, and other genes directing histone methylation, has been shown to modify H3K4 and regulate healthspan in *C.elegans* (Greer and Brunet, 2010). Greer and Brunet found that excess H3K4 trimethylation is detrimental to lifespan.

Because histone proteins are among the most highly conserved proteins in eukaryotes, histones play a crucial role in the biology of the nucleus in many organisms. Studying histone modification in *C. elegans* is useful because results would likely translate across species to human histone regulation.

Hierarchy of chromosome composition: DNA→histone→chromatin→nucleosome→chromosome

List of Genes Being Investigated and Effect on Histone Modification of Chromatin

Strain Name	Suppressed Gene	Function on modification of chromatin
RB1304	*wdr*-5.1	Histone trimethylation of H3K4
MT14911	*set*-2	Methyltransferase. Histone trimethylation/ methyltransferase of H3K4
RB1025	*set*-4	Methyltransferase. Histone trimethylation of H3K4
MT16426	*set*- 9	Methyltransferase. Histone methylation of H3K4
RB2406	*set*-15	Methyltransferase. Histone methylation of H3K4
C767	*set*-18	Methyltransferase. Histone methylation of H3K4
RB777	*hcf*-1	Binds to *daf*-16 and prevents transcription. Histone trimethylation of H3K4

Table 2: Knock down of these genes modulates methylation of histone 3 at lysine 4 (H3K4). Decreased methylation is associated with an altered chromatin state and a slowing of the ageing process.

Although all the genes being investigated function in histone modification, there are unique mechanisms by which each contributes to methylation or cell development. RNAi-mediated knock down of gene *wdr*-5.1 decreases histone methylation, increases transcription, and extends lifespan (Greer and Brunet, 2010). Knockdown of *set*-2, *set*-4, set-9, set-15, and set-18 disables methyltransferase mechanisms (Greer and Brunet, 2010). Knockdown of *hcf*-1 increases expression of *daf*-16. *Hcf*-1 forms a complex with *daf*-16 in the nucleus by binding to various transcription and chromatin factors. Physical association of *hcf*-1 and *daf*-16 reduces the expression of *daf*-16. *Daf*-16 is an effector of the insulin-like growth factor pathway which functions to regulate lifespan through development, metabolism, and stress response (Lee et al., 2008) and (Hamilton et al., 2013). *Hcf*-1 has also been shown to recruit methyltransferase set-1 (Narayanan et al., 2007).

Methods

Standard Conditions for Nematode Growth

To ensure standard conditions for all experiments, *C. elegans* were kept on a 6 cm diameter plate of nematode growth medium (NGM) agar stored at a constant temperature of 20^{o} C. Mutant worms were fed *Escherichia coli* strain OP50 plated at a concentration of $1x10^{9}$ cells/ml. In RNAi experiments, *E. coli* strain HT 115 containing the appropriate vector expressing dsRNA of the genes of interest is used in place of OP50 (Greer and Brunet, 2010). RNAi plates were spotted with ampicillin (100 micro grams/ml working concentration) and isopropylthiogalactoside (IPTG 1 mM working concentration) to prevent contamination and encourage RNA polymeration.

Gene Sequencing

Before application in the experiment, the sequence of each RNAi strain was confirmed through genome sequencing performed by a company called ACGT in Wheeling, IL. A technique called direct colony sequencing was used where bacterial colonies containing the RNAi-producing plasmid were amplified, and then the DNA plasmid was extracted and sequenced. This step ensured that the gene of interest was present in the plasmid contained in the cells of the culture obtained from the Ahringer library.

Determining Age

It was crucial to this experiment to obtain data from worms that were the same age. To acquire an age-synchronous population, adult worms for each strain were placed on a plate spotted with OP50. After eggs were apparent on the plate, the population was treated with a hypochlorite solution composed of 7ml saline solution S. basal, 2 ml Na hypochlorite, and 1 ml 5N KOH for approximately 10 minutes. This hypochlorite solution killed the adult worms and any larvae but did not affect the eggs. The eggs were washed and transferred to fresh plates. All animals hatching from eggs surviving after the hypochlorite treatment were age-synchronized and could be used for the experiment.

Observation

Worms enter adulthood when they begin laying eggs, which begins 3-4 days after hatching. For the experiments performed for this project, a worm's lifespan was recorded starting the first day of adulthood. Worms were manually transferred to fresh plates daily using a small platinum wire that serves as a worm pick. The pick was sterilized by flame between plates to prevent cross-contamination. Worms were scored and transferred daily until they stop laying eggs. After the reproductive stage of life was over, worms were scored every day but transferred to a new plate 3 times a week or earlier if contamination on the plate was apparent.

Healthspan Classification

There are many ways to assess the health of an organism. Movement, appearance, and fertility are all criteria that can be used to evaluate well-being. In the experiments performed for this project, health was measured by observed movement. Since many organisms become physically slower as they

age, measuring movement was an appropriate and non-invasive approach to quantify the ageing process. Each day, worms were individually observed for 1 minute before being transferred. After observation, they were classified into one of four categories (Herndon et al., 2002). As the worms age, they move through the four movement classifications. Healthy worms move spontaneously or if provoked. Class C is considered to be the beginning of morbidity. Once a worm has entered class C death usually occurs within a couple of days. The classification system is important to this experiment because it is a way to quantify the health of *C. elegans* over the lifespan of the worm. Worms that are considered to have a long healthspan spend less time in Class C than worms with a short healthspan.

Spontaneous movement.	The nematode can be observed moving across the plate. Tracks may be present behind the nematode. If the nematode is not moving forward, it can bend the majority of its body right, left, and in an "omega turn". The nematode performs several body bends during the duration of observation.
Provoked Movement	The nematode is stationary until prodded with a platinum pick. After being provoked, the nematode moves forward or backward or begins body bends. Tracks may be present behind the nematode.
Class C	The nematode is not dead, but is not moving freely and barely moves when provoked. When it moves, typically only the anterior of the worm is mobile. There are no fresh tracks, indicating that the nematode has not moved recently.
Dead	The nematode appears limp and lifeless. It does not move at all even when prodded with a worm pick.

Table 3: Illustrates movement classifications used to score healthspan in *C. elegans.*

Data Collection In 3 Experimental Groups

Healthspan group A consisted of mutant worms with knockdown of the *wdr*-5.1 gene and *set*-4 gene compared against a wild-type (N2) control. Healthspan group B consisted of wild type worms treated with RNAi-mediated knock down of the *wdr*-5.1 gene, *set*-9 gene, *set*-15 gene, and *set*-18 gene compared to wild type worms treated with empty vector (EV). A repeat experiment of Healthspan B was performed. Healthspan group C consisted of wild type worms treated with RNAi-mediated knock down of the *hcf*-1 gene, and *set*-4 gene compared to wild type worms treated with empty vector. A repeat experiment of Healthspan C was performed.

Due to time constraints, healthspan experiments were organized in three groups and data was collected over a course of 6 months. Data from Healthspan Group A was collected between October and November. Data from the first experiment of Healthspan Group B was collected between January and

February. The repeat experiment was conducted between February and March. Healthspan Group C data from the first and repeat experiment were collected simultaneously in March. It is important to clarify that genes were placed into each healthspan group at random, and genes in each group have no association with each other besides that data was collected in the same time frame.

Statistical Analysis

Once data were compiled, two statistical tests were performed to determine if there were significant differences in the progression of healthspan between genes being investigated compared to a control group. A log rank test was run to assess if there were differences in the amount of time each variable spent in each healthspan category compared to other variable. A Cox proportional hazard test was conducted to assess how large the effect size was between variables. Statistical analyses of lifespan and healthspan were performed on Kaplan-Meier survival curves in the program R (Greer and Brunet, 2010). Results from the Kaplan-Meier curve reflect the probability that a fraction of the population will remain in a state over time.

Results

Lifespan Analysis

Each gene was selected because it had been proven to extend lifespan in previous studies performed by Greer and Brunet, (2011), Hamilton et al. (2013), and Lee et al,. (2008). A lifespan assay was performed on each group to assess if lifespan extension occurred. Surprisingly, almost the all results indicated no significant effect on life extension, or in many cases a significantly shorter lifespan compared to the control. There was one exception observed in the *set*-2 gene which exhibited significant life extension compared to the control.

In Healthspan group A, experimental groups did not display life extension. In fact, mutant worms for *wdr*-5.1 and *set*-4 RNAi knock down had significantly shorter lifespans than wild type worms ($p=0.020922$, and $p=0.000544$) respectively. Results from the Cox proportional hazard tests indicate that worms treated with *wdr*-5.1 RNAi were 2.0 times more likely to die before the control, and worms treated with *set*-4 RNAi were 2.7 times more likely to die before the control worms.

In Healthspan group B, experimental groups did not display life extension. Worms treated with *wdr*-5.1, *set*-9, and *set*-18 RNAi were significantly less long-lived than wild type worms treated with empty vector ($p= 0.0321$, $p=0.0154$, and $p=0.0587$) respectively. Worms treated with *set*-18 RNAi showed no significant difference in lifespan compared to worms treated with empty vector. Results from the Cox proportional hazard tests indicate that worms treated with *wdr*-5.1, *set*-9, and *set*-15 RNAi were 1.7, 1.8 and 1.6 times more likely to die before the control worms respectively. Interestingly, the repeat experiment of Healthspan group B provided data that did not indicate a significant difference in lifespan between any of the RNAi variables and the control group.

In Healthspan group C, worms treated with *hcf*-1 RNAi had significantly shorter lifespans than wild type worms treated with empty vector ($p=0.001737$). However, worms treated with *set*-2 RNAi

displayed significantly longer lifespans than wild type worms treated with empty vector (p=0.000271). Results from the Cox proportional hazard test indicate that worms treated with *hcf*-1 RNAi were 2.6 times more likely to die before the control, while worms treated with *set*-2 RNAi were 1.7 times more likely to die after the control worms. In the repeat experiment, worms treated with *hcf*-1 RNAi showed no significant difference in lifespan compared to wild type worms treated with empty vector. Worms treated with *set*-2 RNAi again displayed significantly longer lifespans than wild type worms treated with empty vector ($p=4.47e^{-5}$). Results from the Cox proportional hazard test indicated that worms treated with *set*-2 RNAi in the repeat experiment were 1.7 times more likely to die after the control worms.

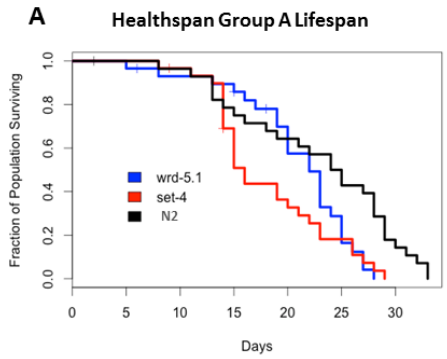
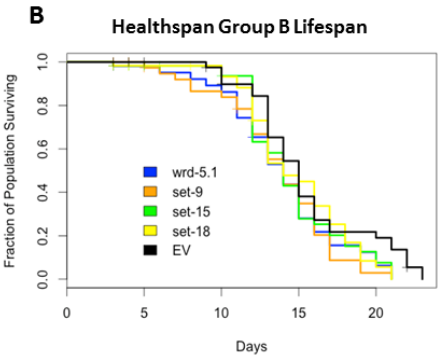
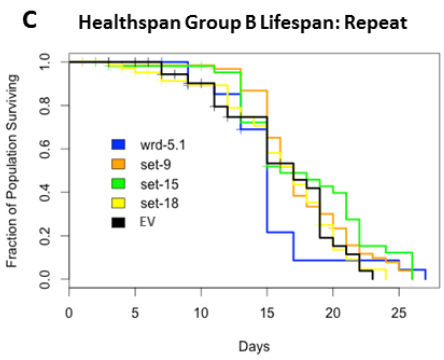
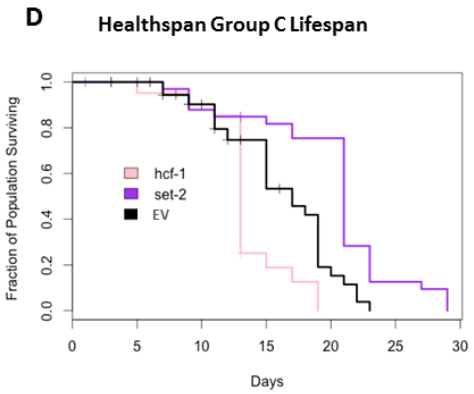
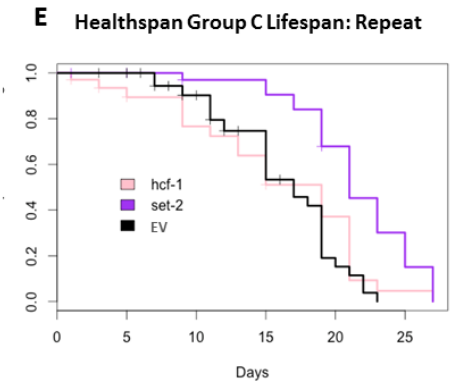


Figure 4 Lifespan. ***A****:wdr-5.1 and set-4 mutants lived significantly fewer days than the control.* ***B****: wdr-5.1, set-9, and set-15 knockdown lived significantly fewer days than the control, there was no difference between set-18 knockdown and the control.* ***C****:In the repeat experiment, there was no significant difference in lifespan between any of the variables.* ***D****: hcf-1 knockdown lived significantly fewer days than the control. Set-2 knockdown was significantly longer lived than the control.* ***E****: In the repeat experiment, there was no significant difference in lifespan of hcf-1 knockdown, but set-2 lived significantly longer than the control*









Healthspan Analysis

Although many of the gens in this experiment did not display the expected phenotype of life extension compared to the control, data collected from scoring healthspan were still considered.

Experimental Healthspan Group A (*wdr*- 5.1 and *set*-4 mutants compared to N2)

In Healthspan group A, worms treated with *wdr*-5.1 and *set*-4 RNAi maintained spontaneous movement for significantly less time compared to worms treated with empty vector (p=0.0198 and p=2.83e^{-11}, respectively). Results from the Cox proportional hazard test indicated that worms treated with *wdr*-5.1 and *set*-4 RNAi were 1.2 and 1.6 times more likely to lose spontaneous movement before the control worms respectively. Worms treated with *wdr*-5.1 and *set*-4 RNAi maintained provoked movement for significantly less time compared to worms treated with empty vector (p=0.00432 and p=1.26e^{-6},respectively). Results from the Cox proportional hazard test indicated that worms treated with *wdr*-5.1 and *set*-4 RNAi were 1.3 and 1.6 times more likely to lose provoked movement before the control worms respectively. Worms treated with *wdr*-5.1 and *set*- 4 RNAi maintained the stage-C phenotype before death for significantly less time compared to worms treated with empty vector (p=0.000208 and p=0.011252, respectively). Results from the Cox proportional hazard test indicated that worms treated with *wdr*-5.1 and *set*-4 RNAi were 2.3 and 1.7 times more likely to lose spontaneous movement before the control worms, respectively.

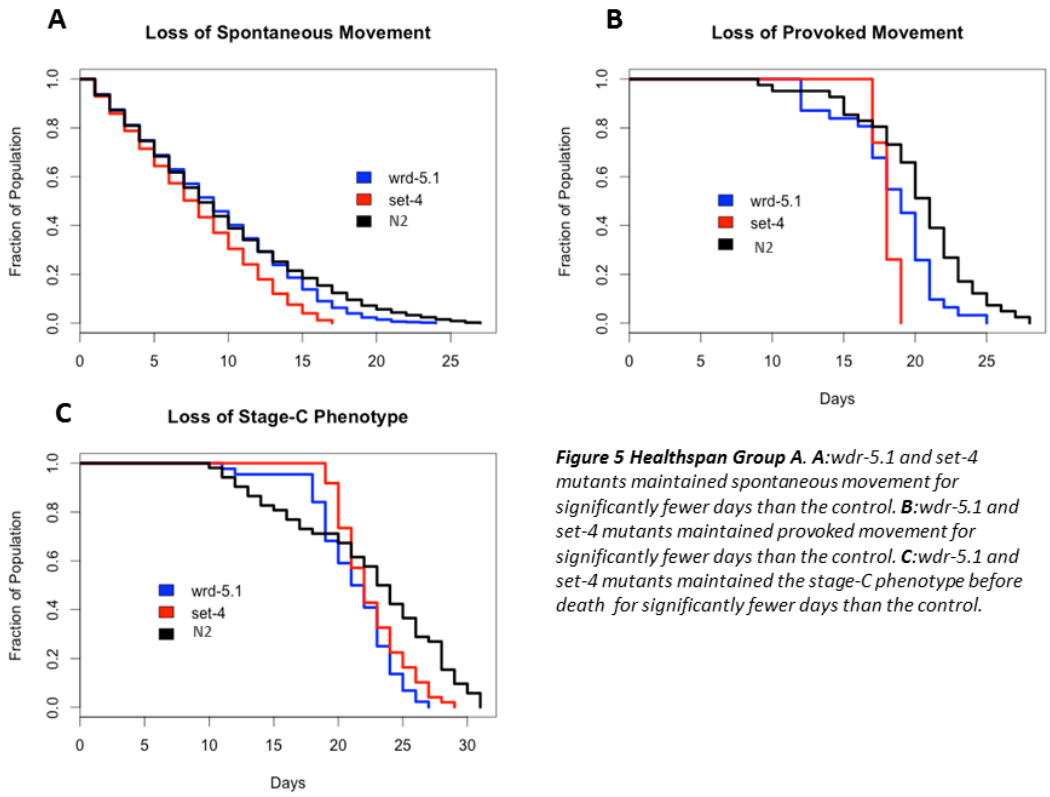


***Figure 5 Healthspan Group A. A**:wdr-5.1 and set-4 mutants maintained spontaneous movement for significantly fewer days than the control. **B**:wdr-5.1 and set-4 mutants maintained provoked movement for significantly fewer days than the control. **C**:wdr-5.1 and set-4 mutants maintained the stage-C phenotype before death for significantly fewer days than the control.*

Experimental Healthspan Group B (*wdr*-5.1, *set*-9. *set*-15, and *set*-18 RNAi compared to EV)

In Healthspan B, worms treated with *wdr*-5.1, *set*-9, *set*-15, and *set*-18 RNAi maintained spontaneous movement for significantly less time compared to worms treated with empty vector ($p=5.53e^{-8}$, $p=2.63e^{-4}$, $p=1.36e^{-10}$, and $p=3.26e^{-8}$, respectively). Results from the Cox proportional hazard test indicated that worms treated with *wdr*-5.1, *set*-9, *set*-15, and *set*-18 RNAi were 1.4, 1.6, 1.5, and 1.4 times more likely to lose spontaneous movement before the control worms, respectively. In the repeat experiment**,** worms treated with *wdr*-5.1 and *set*-9 RNAi displayed no significant difference in the loss of spontaneous movement over time compared to worms treated with empty vector (p=0.54487 and p=0.21672, respectively). Worms treated with *set*-15, and *set*-18 RNAi maintained spontaneous movement for significantly less time compared to worms treated with empty vector (p=0.00123 and $p=1.08e^{-5}$, respectively). Results from the Cox proportional hazard test indicated that worms treated with *set*-15, and *set*-18 RNAi were 1.2 and 1.3 times more likely to lose spontaneous movement before the control worms, respectively.

Worms treated with *wdr*-5.1, *set*-9, *set*-15, and *set*-18 RNAi maintained provoked movement for significantly less time compared to worms treated with empty vector (p=0.000342, p=0.001842, p=0.000503, and p=0.048613, respectively). Results from the Cox proportional hazard test indicated that worms treated with *wdr*-5.1, *set*-9, *set*-15, and *set*-18 RNAi were 3.2, 2.9, 2.9, and 1.9 times more likely to lose provoked movement before the control worms, respectively. In the repeat experiment**,** worms treated with *wdr*-5.1, *set*-9, and *set*-18 RNAi maintained provoked movement for significantly less time compared to worms treated with empty vector (p=0.01077, p=0.024444, and p=0.00191, respectively). Results from the Cox proportional hazard test indicated that worms treated with *wdr*-5.1, *set*-9, and *set*-18 RNAi were 2.5, 2.0, and 2.7 times more likely to lose provoked movement before the control worms, respectively. Worms treated with *set*-15 RNAi displayed no significant difference in the loss of provoked movement over time compared to worms treated with empty vector (p=0.80479).

Worms treated with *wdr*-5.1 and *set*-9 RNAi maintained the stage-C phenotype before death for significantly less time compared to worms treated with empty vector (p=0.03082 and p=0.00502, respectively). Results from the Cox proportional hazard test indicated that worms treated with *wdr*-5.1 and *set*-9 RNAi were 1.8 and 2.1 times more likely to lose stage-C phenotype before the control worms, respectively.Worms treated with *set*-15 and *set*-18 RNAi displayed no significant difference in the loss of the stage-C phenotype before death compared to worms treated with empty vector (p=0.08128 and p=0.11276, respectively)y. The repeat experiment of Healthspan group B provided data that did not indicate a significant difference in display of stage-C phenotype between any of the RNAi variables and the control group. When compared to the control, the p-value for worms treated with *wdr*-5.1 RNAi was (p=0.5878, p=0.0869, p= 0.9303, and p= 0.0774) respectively.

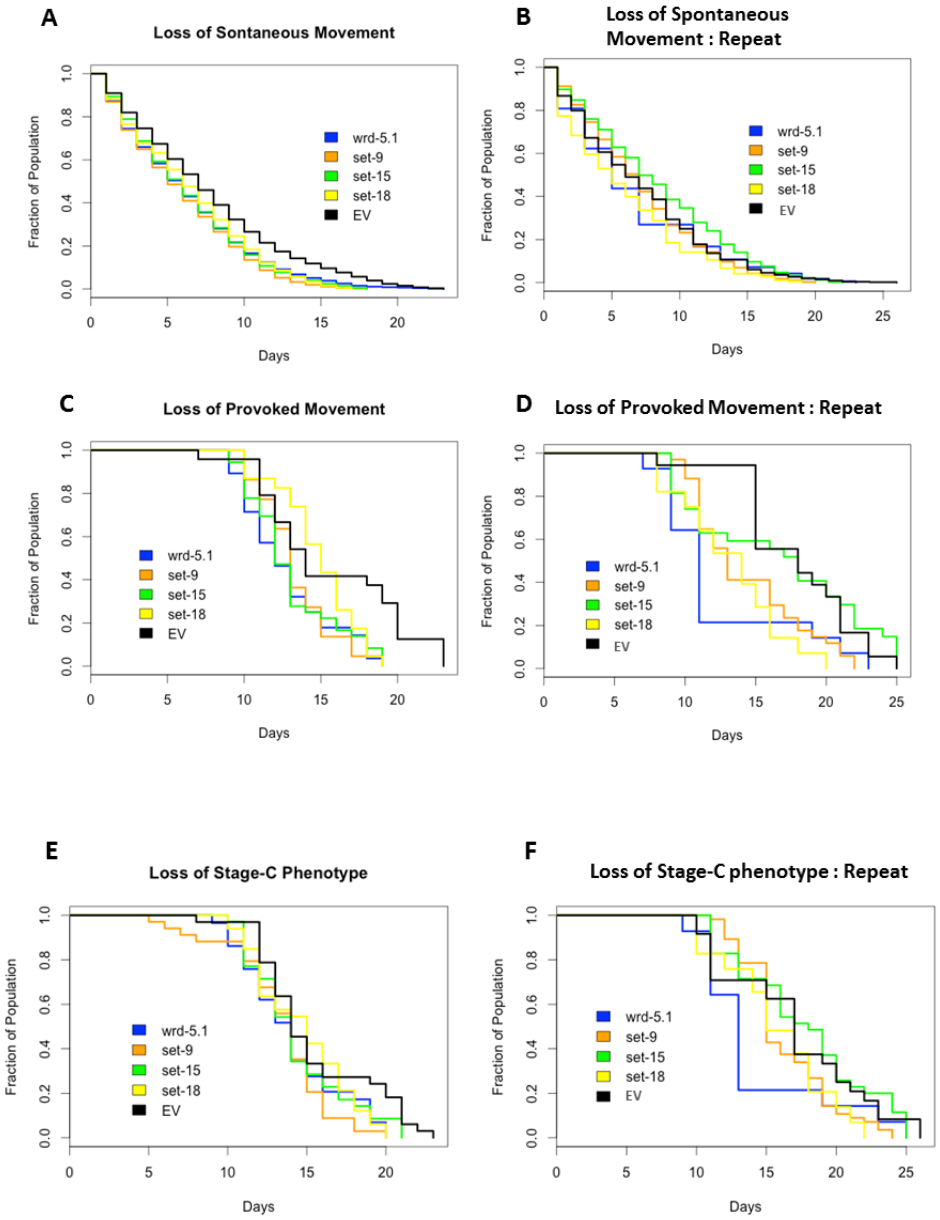


Figure 6 Healthspan Group B. A*:All knockdown variables maintained spontaneous movement for significantly less time than the control.* ***B****: In the repeat experiment, wdr-5.1 and set-9 knockdown maintained spontaneous movement for significantly less time compared to the control. Set-15, and set-18 knockdowns showed no significant difference compared to the control.* ***C****: All variables maintained provoked movement for significantly less time than the control.* ***D****: In the repeat experiment, set-15 knockdown displayed no significant difference in maintenance of provoked movement compared to the control. Wdr-5.1, set-9 and set-18 knockdown maintained provoked movement for significantly fewer days than the control.* ***E****: Wdr-5.1 and set-9 knockdown maintained the stage-C phenotype before death for significantly fewer days than the control. Set-15 and set-18 knockdown displayed no difference compared to the control.* ***F****:In the repeat experiment, there was no significant loss in stage-C phenotype compared to the control.*

Experimental Healthspan Group C (*hcf*-1 and *set*-2 RNAi compared to EV)

In Healthspan group C, worms treated with *hcf*-1 RNAi maintained spontaneous movement for significantly less time compared to worms treated with empty vector ($p=1.268e-^{5}$) and were 1.3 times more likely to lose spontaneous movement then the control. Worms treated with *set*-2 RNAi displayed no significant difference in loss of spontaneous movement over time compared to worms treated with empty vector (p=0.5000). In the repeat experiment, worms treated with *hcf*-1 RNAi displayed no significant difference in loss of spontaneous movement over time compared to the worms treated with empty vector (p=0.37941). Worms treated with *set*-2 RNAi maintained spontaneous movement for a significantly longer period of time time compared to worms treated with empty vector (p=0.00246). Results from the Cox proportional hazard test indicated that *set*-2 knockdown worms were 1.21 times more likely to maintain spontaneous movement compared to the control worms, respectively.

Worms treated with *hcf*-1 RNAi maintained provoked movement for significantly less time compared to worms treated with empty vector ($p=4.81e-^{5}$), and were 9.0 times more likely to lose provoked movement than the control. Worms treated with *set*-2 RNAi displayed no significant difference in loss of provoked movement over time compared to worms treated with empty vector (p=0.0692). In the repeat experiment, similar results were observed. Worms treated with *hcf*-1 RNAi maintained provoked movement for significantly less time compared to worms treated with empty vector (p=0.044), and were 2.0 times more likely to lose provoked movement compared to the control. Worms treated with *set*-2 RNAi displayed no significant difference in loss of provoked movement over time compared to worms treated with empty vector (p=0.341).

Worms treated with *hcf*-1 RNAi maintained the stage-c phenotype before death for significantly less time compared to worms treated with empty vector (p=0.00538) and were 3.3 times more likely to lose the stage-C phenotype than the control. Worms treated with *set*-2 RNAi displayed no significant difference in loss of stage-C phenotype before death over- time compared to worms treated with empty vector (p=0.15906). However, in the repeat experiment, worms treated with *hcf*-1 RNAi displayed no significant difference in loss of stage-C phenotype before death over- time compared to worms treated with empty vector (p=0.853). Worms treated with *set*-2 RNAi also displayed no significant difference in loss of stage-C phenotype before death over- time compared to worms treated with empty vector(p=0.650).

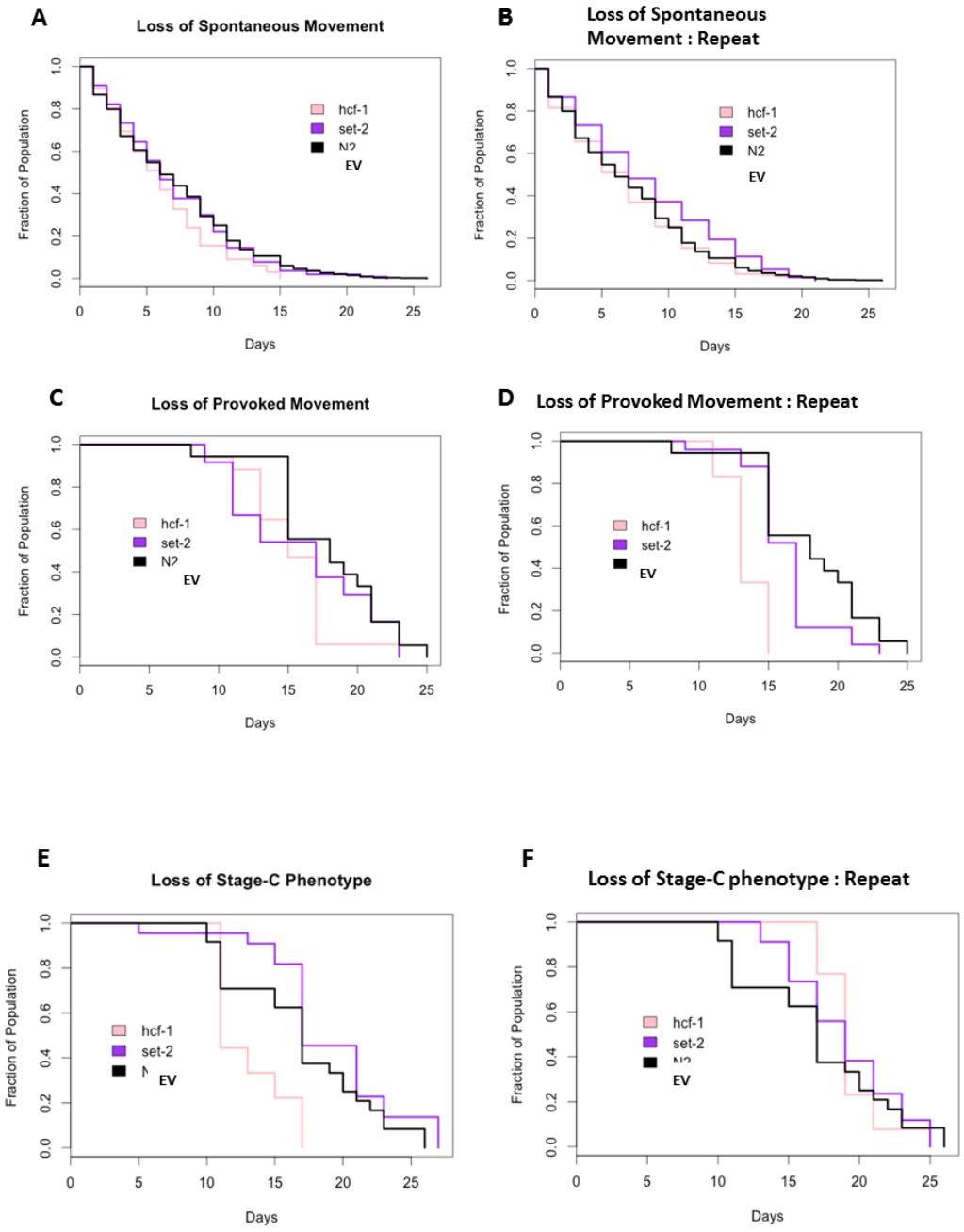


Figure 7 Healthspan Group C. A: *Hcf-1 knockdown maintained spontaneous movement for significantly fewer days than the control. There was no difference between set-2 knockdown and the control.* ***B:*** *In the repeat experiment, there was no difference between hcf-1 and the control. Set-2 knockdown maintained spontaneous movement for significantly longer compared to the control* ***C:*** *Hcf-1 knockdown maintained provoked movement for significantly less time compared to the control. There was no difference between set-2 and the control.* ***D:*** *In the repeat experiment, Hcf-1 knockdown maintained provoked movement for significantly less time compared to the control. There was no difference between set-2 and the control.* ***E:****Hcf-1 knockdown maintained the stage-C phenotype before death for significantly fewer days compared to the control. There was no significant difference between set-2 knockdown and the control* ***F:****In the repeat experiment, neither variable displayed a significant loss in stage-C phenotype compared to the control.*

Summary of Results

Gene (and source of knockdown)	Life-span compared to control	Loss of spontaneous movement compared to control	Loss of provoked movement compared to control	Loss of stage-C phenotype compared to control
Wdr-5.1 (mutant)	Shorter p=0.020922 Coef= 2.0	Likely before P=0.0198 Coef=1.2	Likely before P=0.00432 Coef=1.3	Likely before P=0.000208 Coef=2.3
Set-4 (mutant)	Shorter p=0.000544 Coef=2.8	Likely before P=2.83e^{-11} Coef=1.6	Likely before P=1.26e-^{6} Coef=1.6	Likely before P=0.011252 Coef=1.7
Wdr-5.1 (RNAi)	Shorter or not different p=0.0321 p=0.5878 Coef=1.7	Likely before or no difference P=5.53e^{-8} Coef=1.4 P=0.54487	Likely before P=0.000342 Coef=3.2 P=0.01077 Coef=2.5	Likely before or no difference P=0.03082 Coef=1.8 P=0.05878
Set-9 (RNAi)	Shorter or not different p=0.0154 p=0.2384 Coef=1.8	Likely before or no difference p=2.63e^{-4} coef=1.6 p=0.21672	Likely before P=0.001842 Coef=2.9 P=0.02444 Coef=2.0	Likely before or no difference P=0.00502 Coef=2.1 P=0.0869
Set-15 (RNAi)	Shorter or not different p=0.0587 p=0.0836 Coef=1.6	Likely before P=1.36e^{-10} Coef=1.5 P=0.00123 Coef=1.2	Likely before or no difference P=0.000503 Coef=2.9 P=0.80479	No difference P=0.08128 P=0.9303
Set-18 (RNAi)	Not different P=0.126 P=0.9266	Likely before P=3.26e^{-8} Coef=1.4 p=1.08e-^{5} Coef=1.3	Likely before P=0.048613 Coef=1.9 P=0.00191 Coef=2.7	No difference P=0.11276 P=0.0774
Hcf-1 (RNAi)	Shorter or not different p=0.001737 P= 0.325 Coef=2.6	Likely before or no difference P=2.68e-^{5} Coef=1.3 P=0.37941	Likely before P=4.81e-5 Coef= 9.0 P= 0.0444 Coef= 2.0	Likely before or no difference P=0.00538 Coef=3.3 P=0.853
Set-2 (RNAi)	Longer p= 0.00027 Coef=1.65 p= 4.73e-^{5} Coef=1.70	Likely after or no difference P=0.5 P= 0.00246 Coef= 1.21	No difference P=0.0692 P=0.341	No difference P=0.15906 P=0.650

Table 4: Listed p-values for original experiments and repeat testing. Values for original experiment are listed first, and repeat tests second. Coef= exponential coefficient from proportional hazards test. Value represents the likelihood of event represented by transition from one healthspan classification to the next. If p-values were insignificant, the exponential coefficient was not included.

Discussion

Speculation On Why Life Extension Did Not Occur

Most of the results obtained from the lifespan assay did not match the expectation based on observed life extension in studies performed by Greer and Brunet, (2010), Hamilton et al., (2013), and Lee et al., (2008). The only exception was life extension displayed in both the original experiment and the repeat testing of RNAi knock down of the *set*-2 gene. There are several explanations that may account for the absence of life extension in all gene knockdown variables apart from *set*-2. First, although every effort was made to ensure that methods were identical to those cited by Greer and Brunet, (2010), it is possible that there was an overlooked difference in agar plates the worms were kept on, transfer technique or frequency of transfer of worms, technique for achieving an age synchronous population, or any other number of variables that may have contributed to the absence of effect. Another possible explanation for the absence of life-extension is ambiguity regarding the wild-type *C. elegans* used for RNAi treatment and as a control. It is assumed that wild-type worms used in different laboratories are almost genetically identical, however sometimes mutations occur and produce different strains of wild-type worms. The wild-type used in this project were N2, but other strains include RRF3 and ER11. It is possible that the wild-type strain used by any of the studies preceding this project were dissimilar from N2 and the difference was responsible for incompatible results. Finally, in the 2010 Greer and Brunet study, *wdr*-5.1 and *set*-4 mutants were backcrossed to ensure that they were heterozygous at the loci of the mutation of interest. *Wdr*-5.1 and *set*-4 mutants were not back crossed in this experiment and were homozygous, and this difference may have caused the significantly shorter lifespan compared to the control.

While unlikely, since three major studies support the life-extending effects of each gene investigated, it is necessary to note that a possible reason why no life extension occurred is because knockdown of the genes in question does not produce a prolonged lifespan.

Interpretation of Results

Healthspan Group A

Present morbidity

3) Compression of Morbidity

Healthspan group A worms were homozygous mutants for *wdr*-5.1 and *set*-4 alleles. Both *Wdr*-5.1 and *set*-4 mutants lost spontaneous and provoked movement in significantly fewer days on average compared to the wild-type worms. Since spontaneous and provoked movement are indicative of a

healthy state, loss of these phenotypes early on suggests poor healthspan. *Wdr*-5.1 and *set*-4 mutants entered and left the stage-C phenotype significantly sooner on average than the wild-type worms. The *wdr*-5.1 and *set*-4 mutants died in fewer days than the control worms, but also maintained stage-C phenotype for fewer days on average than the control worms. Because the stage-C phenotype represents the start of morbidity, the amount of time which the *wdr*-5.1 and *set*-4 mutants entered and remained in stage-C indicates that they display a compression of morbidity from table 1.

Healthspan Group B

Present morbidity

No life-extension or improved healthspan: lifespan ratio

Overall, worms from Healthspan group B displayed either significantly fewer days, or no significant difference in days spent in a healthy state of spontaneous or provoked movement. There was no significant difference in the time spent in a state of morbidity represented by the stage-C phenotype in both experimental groups. For all of the worms in Healthspan group B, lifespan was shorter with no effect on the healthspan:lifespan ratio.

Healthspan group C

Present morbidity

2) Life extension with no change in morbidity (postponed morbidity)

. There was no significant difference between any of the healthspan classification categories demonstrated in both experiments by *hcf-1* knockdown. However, *set*-2 maintained spontaneous movement and was significantly longer lived compared to the control worms. Therefore, knock down of *set*-2 produces worms that display a shift to the right in the healthspan:lifespan ratio represented in Figure 1.

Conclusion

Worms treated with RNAi that mediates the knock down of the *set*-2 gene demonstrated extended lifespan as observed in research conducted by Greer and Brunet. The healthspan:lifespan ratio in these animals was improved because the start of the state of morbidity shifted to the right in proportion to the extension of lifespan.

It is difficult to assess the healthspan of the other genes investigated in this project because none displayed the expected phenotype of life extension observed in studies performed by Greer and Brunet. If healthspan is considered disregarding lifespan, then it can be concluded that mutants for genes *wdr*-5.1 and *set*-4 expressed compression of morbidity while worms treated with RNAi that mediates the knockdown of *wdr*-5.1, *set*-9, *set*-15, and *set*-18 expressed a shorter lifespan, without any change or improvement in healthspan:lifespan ratio.

Limitations and Moving Forward with Healthspan

A major limitation encountered in this study was the unexpected absence of a life extension observed in the subjects. Because the research question was based on the assumption that life extension would occur, it is difficult to clearly conclude what the true relationship of the healthspan:lifespan ratio is for each gene of interest. Another major limitation was time constraints. Ideally, more repeat experiments would be performed to further validate or invalidate findings. For example, there was no repeat experiment performed for Healthspan Group A, and therefore results collected from the data from that group cannot be considered with as much confidence as results from the other healthspan groups. Time constraints were additionally limiting because it was not possible to collect data from each gene of interest within the same time frame. Because it can take up to several hours to score and transfer worms, it was essential, although not ideal, to investigate certain genes at different times instead of all at once. Error that might have occurred from collecting data over different time frames could be avoided if all the gene variables could be investigated at once. A final limitation of this study was sample size. Sample sizes ranged from 30-60 before subjects were even censored from the experiment. An increase in sample size would provide more accurate results. As ageing research and technology continues to advance, the relevance and importance of the healthspan concept will also expand. The lifespan scenarios discussed in this project are crucial to understanding the complexity of the interaction of healthspan and lifespan.

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